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High Susceptibility of Human Dendritic Cells to Invasion by the Intracellular Pathogens *Brucella suis, B. abortus*, and *B. melitensis*

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Bacteria from the *Brucella* genus are able to survive and proliferate within macrophages. Because they are phylogenetically closely related to macrophages, myeloid dendritic cells (DCs) constitute potential targets for *Brucella* bacteria. Here we report that DCs display a great susceptibility to *Brucella* infection. Therefore, DCs might serve as a reservoir and be important for the development of *Brucella* bacteria within their host.

Brucella are facultative intracellular bacteria that induce chronic infections in a wide range of mammals, including domestic animals and humans. *Brucella* bacteria are responsible for brucellosis and Mediterranean fever, also known as Malta fever. After invasion of the lymphoid system, the bacteria develop within mononuclear phagocytes, and the infected cells play a crucial role in the dissemination of the bacteria in specific locations of the body (spleen, brain, heart, and bones). Of intramacrophagic pathogens, *Brucella* bacteria are among the most powerful and are able to multiply intracellularly up to several thousandfold. The common ontogeny of macrophages and myeloid dendritic cells (DCs) and the discovery of a primordial role for DCs in immunity have raised the question of a relationship between DCs and intramacrophagic pathogens. Therefore, we have investigated the possible interaction between *Brucella* bacteria and DCs.

Macrophages and immature DCs were prepared from peripheral blood circulating monocytes obtained by centrifugation on Ficoll-Hypaque (Sigma, Lyon, France) of buffy coat from healthy donors. Monocytes were purified on a magnetic column using anti-CD14-antibody-conjugated microbeads (Miltenyi-Biotec, Paris, France) and differentiated for 5 days in complete medium (RPMI 1640–10% fetal calf serum) supplemented with 50 μM β-mercaptoethanol, 500 U/ml of interleukin-4, and 1,000 U/ml of granulocyte-macrophage colony-stimulating factor (Immunotools, Friesoythe, Germany) for DCs or with 10^{-7} M vitamin D3 (Hoffman-Laroche, Bale, Switzerland) for syngeneic macrophages. Immature DCs were CD14 null (100%), CD83 null (>95%), DC-SIGN positive (100%), and CD1a positive (100%) and CMH-II low (100%). Macrophages were 100% CD14 positive, CD54 low, CD80 low, and CD86 low. After their differentiation, the cells were infected for 1 h at a multiplicity of infection (MOI) of 50 with the green fluorescent protein (GFP)-expressing strains of three *Brucella* species, *B. suis*, *B. abortus*, and *B. melitensis*, or with different *B. suis* attenuated mutants (Table 1). They were then washed with phosphate-buffered saline (PBS) and reincubated in fresh medium supplemented with 30 μg/ml gentamicin to kill remaining extracellular bacteria. Figure 1A shows the observation by fluorescence microscopy of cells infected with the GFP-expressing *Brucella* spp. (11) at 48 h postinfection (p.i.).

As frequently described, whatever the species considered, *Brucella* bacteria have strongly proliferated inside macrophages. Strikingly, the cytoplasm of DCs was completely invaded, some cells containing several hundred *Brucella* bacteria. Figure 1B compares the intracellular development results for *B. suis* within DCs and syngeneic macrophages. The infected cells were washed and lysed in 0.1% Triton X-100 at several intervals p.i. The intracellular number of viable bacteria (in CFU per well) was determined by plating serial 10-fold dilutions onto tryptic soy agar plates. For both types of cells at 1 to 3 h p.i., we first observed a decrease in bacterial numbers. This decrease could be due to the killing of surface-associated bacteria by gentamicin and/or to the cell bactericidal activity, DCs being, like macrophages, able to produce microbicidal agents (5), reactive oxygen species (6, 34), and, in some cases, nitrogen radicals (4, 6). Then, *B. suis* bacteria began proliferating with equivalent growth rates in the two types of cells. To proliferate within macrophagic cells, *Brucella* bacteria, like other pathogens, have developed several virulence processes. Compared to *B. suis*, their rough mutants display a marked decrease in virulence (in vivo and in vitro) associated with an increased interaction with macrophages (15) and a potent capacity for macrophage-stimulating activity (14), thus defining the role of the lipopolysaccharide O chain in virulence. *B. suis manB*, for instance, a mutant in which the gene encoding phosphomannose mutase ManB is inactivated, is greatly attenuated (9).

Two other principal virulence systems which have been identified are the type IV secretory system VirB (3, 26) and the two-component system BvrR/BvrS. Their respective mutants isolated from *B. suis* (3, 20) and *B. suis manB* were tested for their ability to invade DCs. Figure 1C and D show that all three mutants were attenuated in DCs, with a rapid and marked elimination of *B. suis manB* and an inability of the two others to develop intracellularly, as seen with macrophages (15, 20, 26). The similarities in the kinetics of invasion (Fig. 1B) and

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TABLE 1. *Brucella* strains used in the study

Strain	Genotypic and/or phenotypic description (reference)
B. suis 1330	Smooth wild-type *Brucella suis* (ATCC 23444)
B. suis GFP	Smooth ampicillin resistant mutant of *B. suis* 1330 which constitutively expresses gene of GFP (11)
B. abortus GFP	Smooth ampicillin resistant mutant of *B. abortus* 2308 which constitutively expresses gene of GFP (11)
B. melitensis GFP	Smooth ampicillin resistant mutant of *B. melitensis* 16M which constitutively expresses gene of GFP (11)
B. suis manB	*B. suis manB*::mTn*5*Km2 rough mini-Tn*5* insertion mutant of *B. suis* 1330 invalidated on phosphomannose gene, kanamycin resistant (9)
B. suis virB5	*B. suis virB5*::Kan smooth insertion mutant of *B. suis* 1330 (3)
B. suis bcsp31::GFP	Smooth nalidixic acid-resistant mutant of *B. suis* 1330 which carries a promoter-*GFP* fusion inserted into the *bcsp31* gene (20)
B. suis bvrR	*B. suis bcsp31*::GFP, *bvrR*::mTn*5*Km2 smooth Mini-Tn*5* insertion mutant of *B. suis bcsp31*::GFP invalidated into *bvrR* gene, kanamycin resistant (20)

the requirement for *manB*, *virB*, or *bvrR* genes were in line with a common virulence strategy of *Brucella* spp. during the expansion phase of the bacteria in macrophages and DCs. However, when infections were performed under similar conditions, the numbers of intracellular viable bacteria were markedly higher in DCs than in macrophages. This could result from a higher bactericidal activity of the macrophages (5) or a higher uptake of *Brucella* bacteria by DCs or possibly a combination of both phenomena.

To address this issue, we analyzed the phagocytosis of *Brucella* spp. at the onset of infection in the two types of cells. Phagocytic activities of macrophages and immature DCs have not often been compared; moreover, conflicting conclusions have been reported (17, 22, 31). Macrophages and DCs were infected with *B. suis*; in parallel, the phagocytosis of 1 μm latex beads or *B. suis manB* was analyzed as a control. Experiments were performed with different bacterium (or bead)/cell ratios to characterize precisely the dynamics of interaction. Figure 2 shows the results obtained by flow cytometry; similar conclusions ensue from experiments performed using fluorescence microscopy (data not shown). For the measurement of levels of cell-associated bacteria by flow cytometry, 10^5 DCs or syngeneic macrophages were cultured in the presence of *B. suis*, *B. suis manB*, or fluorescein isothiocyanate (FITC)-stained latex beads at the indicated bacterium (or bead)/cell ratio and incubated for 1 h at 37°C in complete medium. After five washes with PBS–5 mM EDTA to eliminate the bacteria or particles not associated with cells, the cells were resuspended in the same buffer and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, le Pont de Claix, France). The GFP (or FITC)-staining measurement allowed us to analyze the percentages of cells which were associated with bacteria or beads. In conditions under which beads (Fig. 2C) and *B. suis manB* (Fig. 2B) were equivalently associated with DCs and macrophages, *B. suis* clearly showed a greater (15- to 20-fold) rate of association with DCs than with macrophages (Fig. 2A). The differences in penetration could mean that the invasion pathways differed. Data on cell surface receptors implicated in *Brucella* phagocytosis are contradictory and not universally accepted (7, 8, 19, 29, 32). Nevertheless, a consensus exists which implicates lipid rafts in penetration of *Brucella* bacteria inside macrophages (1, 18, 19, 23, 28, 33). Therefore, the importance of these structures in DC invasion was assayed.

Infections were performed in the presence of microdomain-disrupting drugs by binding or depletion of cholesterol (filipin [Fig. 3A, B, and C] and β-methylcyclodextrin [Fig. 3D and E]). The cells were pretreated for 30 min, the drugs remaining present during infection. Percentages of infected cells were determined as described previously, both by fluorescence microscopy and flow cytometry. *B. suis manB* and latex beads were used as references for raft-independent phagocytosis (Fig. 3B, C, and E). Parallel experiments with syngeneic macrophages allowed control of the drug efficiencies (Fig. 3, open circles). For an optimum dose of inhibitors, which did not affect cell viability, *Brucella* penetration in DCs was inhibited up to 50% with β-methylcyclodextrin and 70% with filipin, demonstrating that lipid rafts were involved in the phenomenon. At these drug concentrations, the phagocytosis of *Brucella* bacteria by macrophages was inhibited by 90%. The lower sensitivity of DCs compared to macrophages could originate from differences in structure, composition, or the physiology of lipid rafts. Moreover, because of the elevated number of ingested bacteria and the higher number of infected DCs, higher doses of inhibiting drugs could be required in DCs than in macrophages to block phagocytosis completely. This hypothesis is supported by a total inhibition of *Brucella* phagocytosis by the drugs when DC infection was performed at a lower MOI (data not shown). Recent data have shown that *Brucella* interaction with lipid rafts involves cyclic β-1,2-glucan (1), a bacterial annular oligosaccharide able to encage cholesterol. This molecule synthesized by the bacteria, which targets the cellular lipidic microdomains, is required for subsequent mechanisms such as the inhibition of phagolysosome fusion and is finally crucial for *Brucella* intracellular proliferation. This suggests that the interaction between *Brucella* bacteria and lipid rafts is defined by a bacterial property and is necessary for *Brucella* development whatever the host cells considered. Therefore, our data showing that *Brucella* bacteria which proliferated in DCs used lipid rafts to penetrate into these cells agree with this proposal.

We thus hypothesized that differences in *Brucella*-host interaction before penetration could account for the observed differences in DC and macrophage invasion and explored these early interactions by scanning electron microscopy. DCs and macrophages were infected as described previously for 15 or 30 min with *B. suis* or *B. suis manB* (MOI = 100) or with beads (beads/cell = 20). After extensive washing, the cells were fixed with 2.5% glutaraldehyde for 1 h and then progressively dehydrated using a graded (30 to 100%) ethanol series. Subsequently, the samples were critical point dried in CO_2, coated

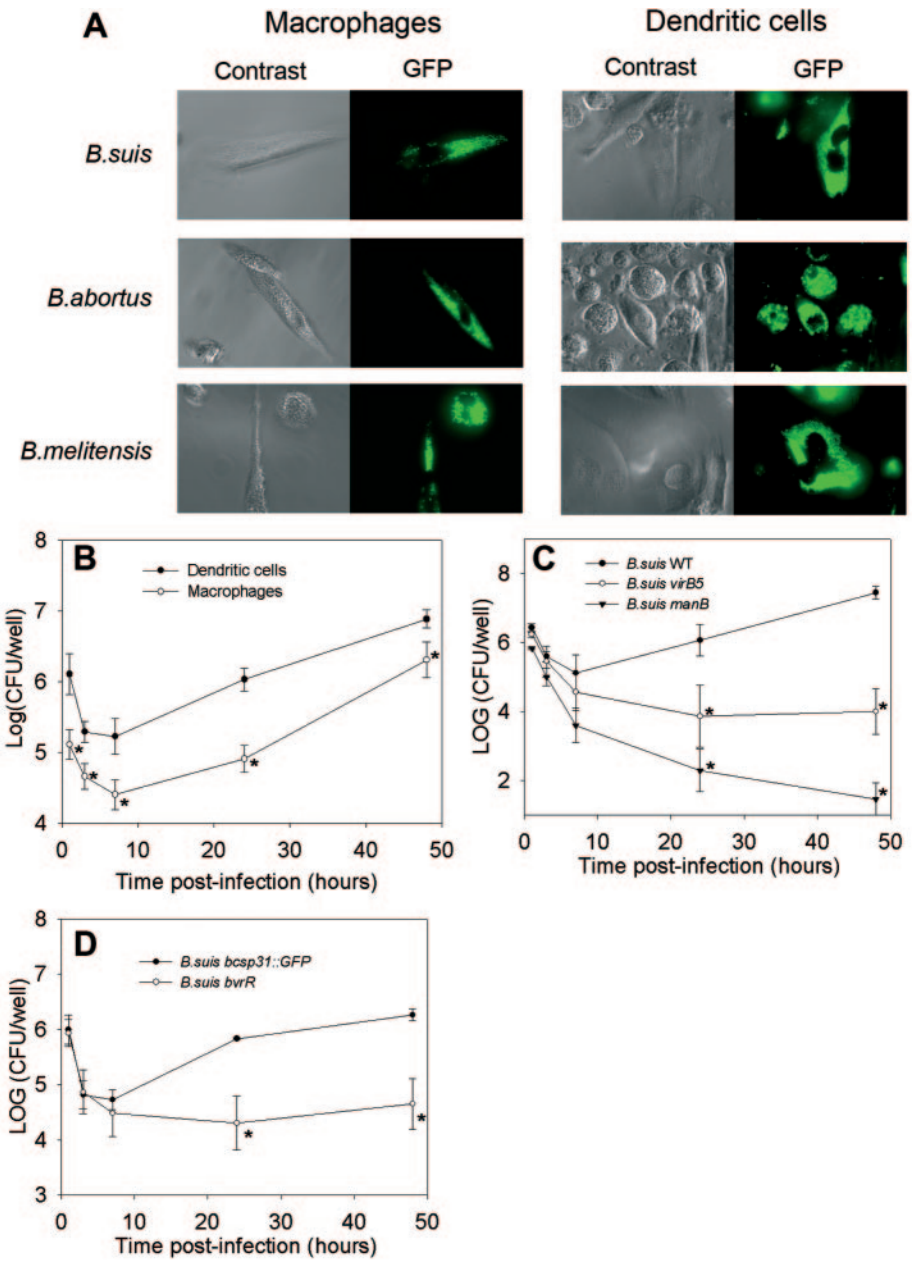

FIG. 1. Intracellular proliferation of *Brucella* spp. (A) A total of 10^5 macrophages or DCs infected with *B. suis* GFP, *B. abortus* GFP, *or B. melitensis* GFP were cultured on eight-chamber Labtek slides in gentamicin-containing medium. At 48 h after the onset of infection, the green fluorescence of the *Brucella* GFP was visualized by videomicroscopy. Results are representative of five different experiments. (B) A total of 10^5 DCs and macrophages were infected with *B. suis* GFP in 48-well plates. The intracellular development of bacteria was then followed by measuring the number of CFU/well at different times p.i. Data are means ± standard errors of the means (SEM) of 18 different experiments. (C) DCs were infected with the *B. suis* WT or the indicated mutants. The intracellular development of bacteria was then followed by measuring the number of CFU/well at different times p.i. Data are means ± SEM of three independent experiments. (D) DCs were infected with *B. suis bvrR* or control strain *B. suis bcsp31*::GFP. The intracellular development of bacteria was then followed by measuring the number of CFU/well at different times p.i. Data are means ± SEM of four independent experiments. *, differences between DCs and macrophages (B) or mutants and controls (C and D) were statistically significant (i.e., $P < 0.01$, as calculated using a paired *t* test).

with gold-palladium, and examined under a scanning electron microscope (an Hitachi 4000 at CRIC Montpellier, France). After 15 min, numerous DCs presented several bacteria on their surfaces (Fig. 4A). A total of 95.5% (±1.5%) of these bacteria were associated with villosities covering DCs (Fig. 4A and E). In contrast, only isolated bacteria could be detected in contact with a small number of macrophages (Fig. 4I), and these did not seem to be associated with any specific membrane protrusion (96% ± 0.4%) (Fig. 4M). After 30 min of infection, the appearance of DCs was markedly altered. Villi had disappeared, and the cell surface was covered by veils (compare Fig. 4A and B). Veils engulf *Brucella* bacteria in

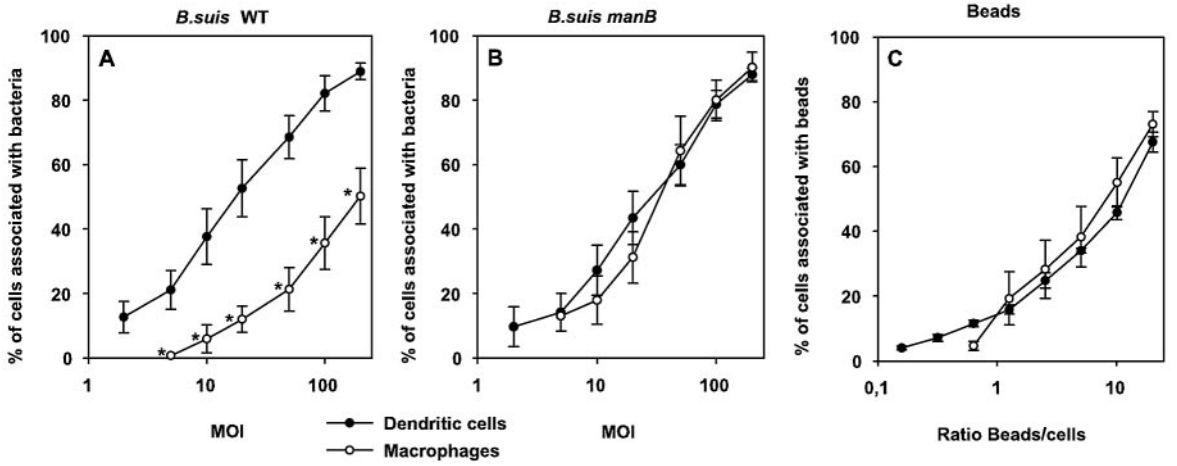

FIG. 2. Comparison of *Brucella* phagocytosis results for dendritic cells and macrophages. Macrophages (open circles) or DCs (closed circles) were cultured in the presence of different bacterium/cell or bead/cell ratios for 1 h. Afterward, cells were washed and resuspended in PBS-EDTA buffer. Percentages of cells associated with GFP-*B. suis* WT (A), GFP-*B. suis manB* (B), or FITC-stained beads (C) were determined by flow cytometry analysis. Data are means ± SEM of six independent experiments. *, differences between DCs and macrophages were statistically significant (i.e., $P < 0.01$, as calculated using a paired *t* test after required data transformations).

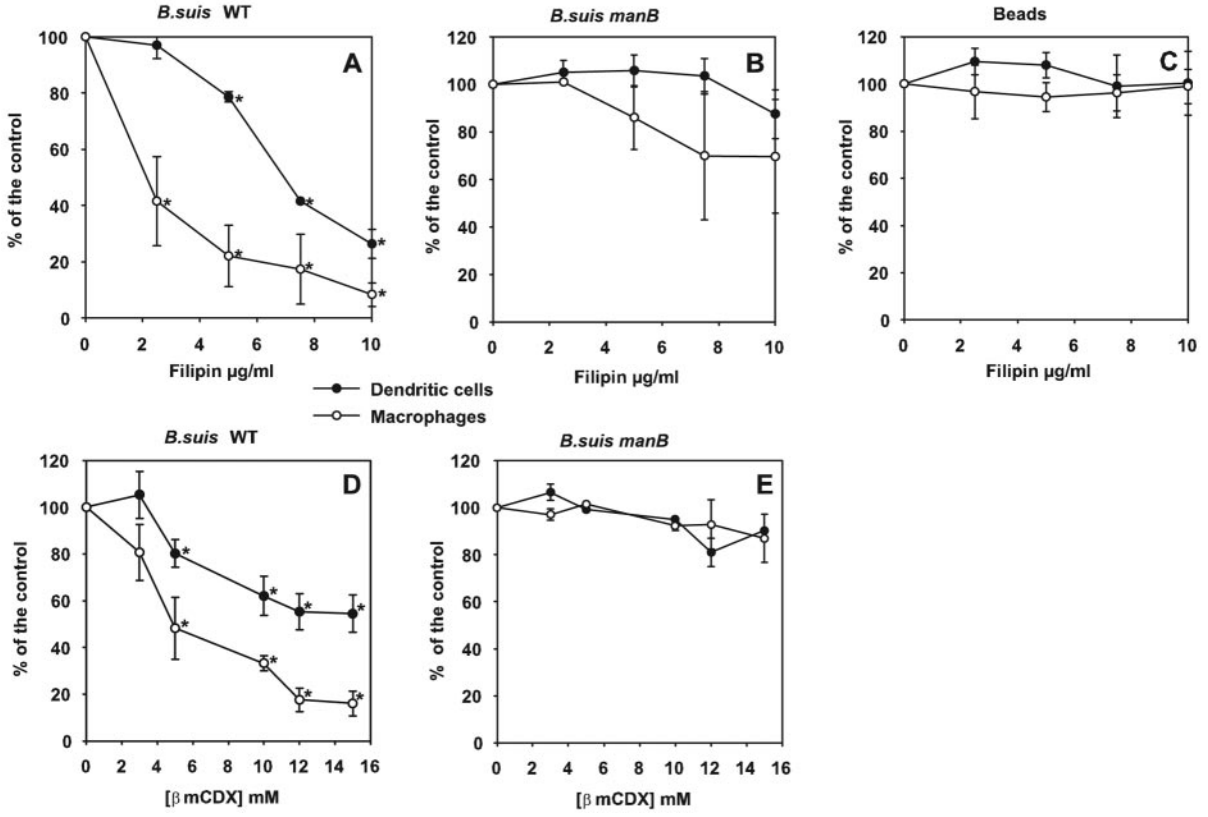

FIG. 3. Implication of lipidic microdomains in *Brucella* phagocytosis. Macrophages (open circles) or DCs (closed circles) were left untreated or were pretreated with filipin (A, B, and C) or β-methyl-cyclodextrin (D and E) for 30 min. They were then infected for 1 h with *B. suis* (MOI = 50) (A and D), *B. suis manB* (MOI = 50) (B and E), or FITC-stained beads (MOI = 10) (C) in the presence of the drugs. The percentages of labeled cells were determined by flow cytometry (A, B, and C) and fluorescence microscopy (D and E). The ratio (percentage of infection in treated cells)/(percentage of infection in untreated cells) was calculated. Data are ratio means ± SEM of three independent experiments. *, differences between treated and untreated cells were statistically significant (i.e., $P < 0,01$, as calculated using a paired *t* test after required data transformations).

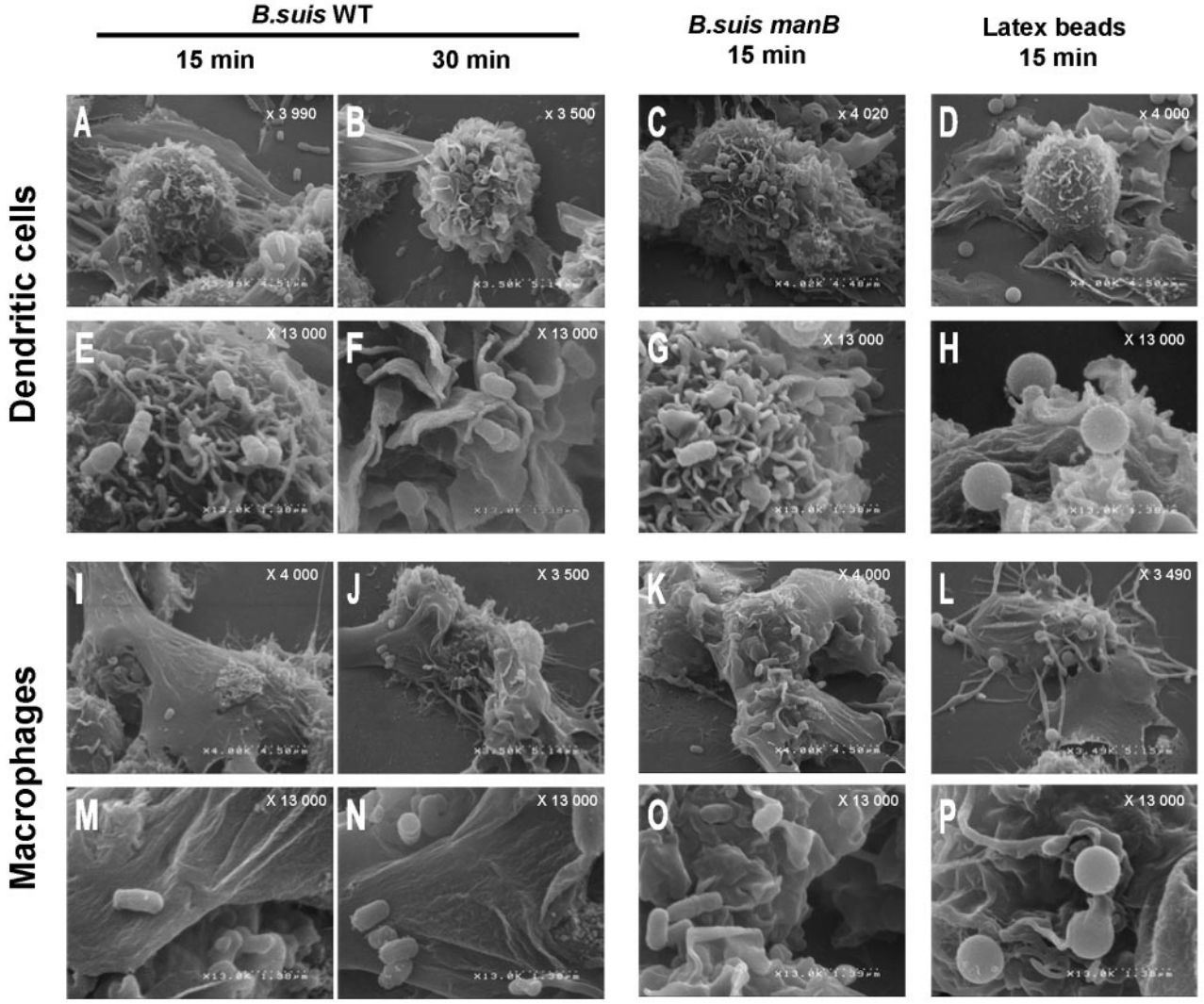

FIG. 4. Visualization of bacterium-cell interaction by scanning electron microscopy. DCs (A to H) or macrophages (I to P) were cultured in the presence of *B. suis* (A, B, E, F, I, J, M, and N), *B. suis manB* (C, G, K, and O), or latex beads (D, H, L, and P) for 15 or 30 min as indicated. Magnifications are indicated on each individual picture. The figure panels are representative of five independent experiments. The percentages of bacteria associated with villosities were evaluated by analyzing more than 200 cells presenting bacteria on their surfaces.

contact with the cells (Fig. 4F). The number of bacteria associated with the macrophage membrane still remained lower than on the DC surface (Fig. 4J). Moreover, no membrane reaction was visible, even in areas where several bacteria were in contact with macrophage membranes (Fig. 4N). Several *B. suis manB* bacteria could be observed on cell surface after only 15 min of contact in both macrophages and DCs (Fig. 4C and K). Bacteria were closely associated with DC or macrophage membranes, which both always showed noticeable reactions once in contact with rough *B. suis manB* (Fig. 4G and Fig. O). Latex beads, which were very efficiently phagocytosed, interacted similarly with the two types of cells, as several beads were observed in association with the cellular surface (Fig. 4D and L). The membrane protrusions developed by DCs and macrophages to capture latex beads were similar (Fig. 4H and P), even when the cell morphologies were different. The extent of the reaction with *B. suis manB* or latex beads confirmed that under our experimental conditions, DCs were fully functional and able to act as professional phagocytes. All together, our data led to the following conclusions. (i) Disparities in infection efficiencies between macrophages and DCs derived from early processes occurring during the initial contact. This could be related to virulence mechanisms of *Brucella* bacteria, since such discrepancies were not observed with *B. suis manB* or latex beads. (ii) The elevated invasion of DCs by *Brucella* bacteria resulted from a specific cellular reaction inducing veil formation, possibly by activating receptors responsible for veil development. Veils could then allow the enhanced capture of bacteria by DCs. Such phenomena did not occur during *Brucella*-macrophage interaction.

The discrepancies in *Brucella* interaction between macrophages and DCs could be interpreted in two opposite ways. (i) *Brucella* bacteria could induce specific cellular processes which enhance the phagocytic activity of DCs compared to that of

macrophages. (ii) *Brucella* bacteria could avoid full contact with the macrophage membrane and thus diminish their phagocytic capability. This would be in line with the stealthiness attributed to *Brucella* bacteria during macrophage infection (16, 21) and could involve both the membrane structures of bacteria (differences between the *B. suis* wild type [WT] and the *manB* mutant) and those of cells (differences between DCs and macrophages).

The present study established some crucial aspects of interactions between *Brucella* bacteria in DC and macrophage infections, which have been extensively studied, thus providing a very interesting point of comparison. *Brucella* interactions with DCs and macrophages present some aspects which were profoundly different and others which were relatively similar. The bacteria have a marked preference for DCs, with a better interaction visible at the level of bacterium/cell contact. Once inside their hosts, *Brucella* bacteria proliferate in similar ways in both cell types. Thus, DCs behave as highly permissive host cells for *Brucella* development. Furthermore, the DC migratory properties which could support *Brucella* bacteria spreading provide an additional potential advantage for the bacteria. Indeed, during establishment within their host, *Brucella* bacteria invade the lymphoid system from the initial site of infection. Migration of DCs from mucosa to lymph nodes could transform these cells into excellent carriers for *Brucella* bacteria.

It is interesting to notice that, among intramacrophagic bacterial pathogens, *Salmonella* (12, 25), *Legionella* (24), and *Chlamydia* (27) spp. are unable to develop within DCs. These pathogens do not succeed in completing their replicative cycle, and they only persist without proliferation or are eliminated. It has been reported that *Mycobacterium* spp. are able to proliferate within murine DCs (2), but this study mentions only a slight multiplication during a very short period of time, considering *Mycobacterium* physiopathology. In contrast, Tailleux et al. have performed experiments over a 1-week period, showing that *Mycobacterium* spp. do not proliferate within human DCs (30), a result in concordance with in vivo observations during mouse infections. Moreover, *Mycobacterium tuberculosis* persists but does not proliferate within splenic DCs (13) whereas it multiplies extensively within spleenic macrophages (10); finally, *Mycobacteria* spp. display an inability to grow within DCs whereas they efficiently invade macrophages.

The ability of *Brucella* bacteria to infect DCs more easily than macrophages and to finalize an extensive multiplication within DCs appears very peculiar. It could be related to a specific virulence strategy and an original intracellular behavior specific to this pathogen. Even if it is not possible to prejudge the role of DC infection in immune system dysfunctions during human brucellosis, the potent infection of DCs raises this issue. Experiments are now in progress to analyze the link between the intracellular proliferation of *Brucella* bacteria in DCs and the inefficiency of specific immune responses during human brucellosis.

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